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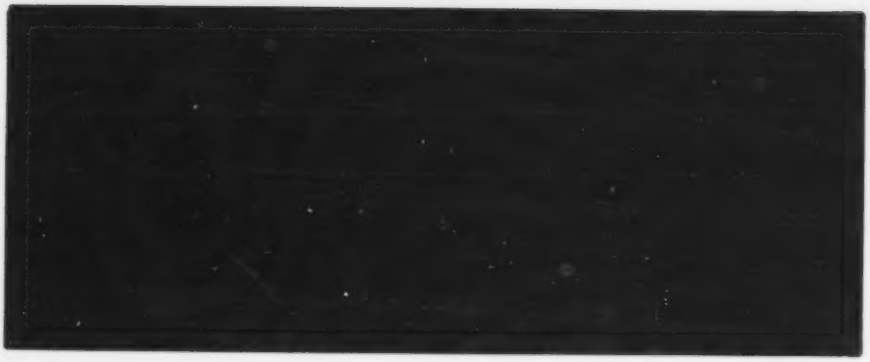

Final Report

2.4-29

Detection of Genetic Polymorphism on Plasma Proteins in Cattle by Immunoblotting

Conducted by
H. Tabel and G. Kraay

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Title: Detection of genetic polymorphism on plasma proteins in cattle by immunoblotting

H. Tabel & G.J. Kraay

FINAL TECHNICAL REPORT

We set out to investigate whether we could determine genetic polymorphism in 3 plasma proteins of the complement system of cattle: factor H, factor B and complement C3. We had to develop techniques to detect such polymorphism. The bovine complement system is a natural defense mechanism against infectious diseases. We succeeded in determining genetic polymorphism in bovine factor H by the combined techniques of SDS-polyacrylamide electrophoresis of bovine plasma and immunoblotting. Three phenotypes (S, SF, F) were identified in a sample population of 149 cattle. Variant S and F differed by an apparent molecular weight of 5,000 daltons. Family studies demonstrated Mendelian segregation of variants S and F. The data indicate that these genetic variants of bovine factor H are encoded by two codominant alleles at a single autosomal locus. The frequency of the allele S in the Holstein parents, Holstein offspring, crossbred parents and crossbred offspring were 0.50, 0.65, 0.75 and 0.62 respectively.

A manuscript entitled: "Genetic polymorphism of complement factor H in cattle" has been accepted by Animal Genetics and will be published early in 1990. A copy of the manuscript is enclosed.

Applying the same techniques we did not detect genetic polymorphism in bovine factor B and C3 on the basis of size differences.

We then turned to a technique employing regular polyacrylamide electrophoresis combined with immunoblotting to detect differences in charge. We spent considerable time on getting this technique to work. The work was hampered by the fact that our competent technician, Heather Groat, left the project to accept a more permanent position. The new technician required considerable time to get familiar with the new work.

Eventually, we succeeded to see differences in factor B from different cattle. The band pattern appeared complex and we ran out of time to establish whether the differences observed had a genetic basis. We intend to pursue this work under the new ADF Project No. R-89-01-0439.

In summary, a method for detecting genetic variants of factor H in cattle has been established. We are confident that we will be able to determine genetic variants for other proteins of the bovine complement system.

This result will be of immediate and long-term benefit:

1) It will be an aid in parentage testing;

2) It is a beginning in characterizing genetic variants of a natural defense mechanisms of cattle against infectious diseases.

Genetic polymorphism of complement factor H in cattle

H. TABEL & H. J. GROAT, *Department of Veterinary Microbiology, University of Saskatchewan, Saskatoon, Canada*
G. J. KRAAY, *Bovine Blood Typing Laboratory, Saskatchewan Research Council, Saskatoon, Canada*
W. P. ASTON, *Department of Microbiology, Queen's University, Kingston, Ontario, Canada*

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Correspondence: Dr. H. Tabel, Dept. of Veterinary Microbiology, University of Saskatchewan, Saskatchewan, S7N 0W0, Canada.

Summary

Bovine factor H was found to be polymorphic by the combined techniques of SDS-polyacrylamide electrophoresis of bovine plasma and immunoblotting. Three phenotypes (S, SF, F) were identified in a sample population of 149 cattle. Variant S and F differed by an apparent molecular weight of 5,000 daltons. Family studies demonstrated Mendelian segregation of variants S and F. The data indicate that these genetic variants of bovine factor H are encoded by two codominant alleles at a single autosomal locus.

Introduction

The alternative pathway of complement is a system of natural resistance to microbial infections (Fearon & Austen 1980; Atkinson & Farries 1987). This system consists of six plasma proteins: complement component C3, factor B, factor D, properdin, factor H and factor I. It can act on microorganisms without the presence of antibodies. By deposition of C3b on the surface of the microbes, it renders microorganisms prone to phagocytosis and lysis. The alternative pathway of complement acts like a cascade of reactions and is self-amplifying. Factor H is a regulatory protein that prevents uncontrolled amplification of the pathway. It binds to C3b and thus (1) regulates the formation of the C3 convertase by displacing factor B, (2) enhances the decay of the C3 convertase by displacing Bb and (3) acts as a cofactor for factor I, the C3b inactivator (Schreiber *et al.* 1978; Pangburn & Müller-Eberhard 1984). There are two major allelic variants of human factor H in Caucasoids with gene frequencies of 0.685 and 0.301 and three minor variants (Rodriguez de Cordoba & Rubinstein 1987).

The structural genes for human factor H and related proteins like C4b-binding protein and the C3b receptor (CR1) form a closely linked cluster termed the "regulators of complement activation" (RCA) cluster (Rodriguez de Cordoba & Rubinstein 1986). The RCA locus has been reported to be in the q32 region of chromosome 1 (Klickstein *et al.* 1987).

Bovine factor H has recently been isolated and found to be a single chain polypeptide of 158,000 daltons (Mhatre & Aston 1987). In this communication, we report the presence of two allelic variants of bovine factor H. They were found to differ in molecular weight by about 5,000 daltons.

Materials and methods

Cattle samples

EDTA blood samples were collected from three different herds of cattle: 39 samples from a pure-bred Holstein herd, including five bulls, and 110 samples from two herds of crossbred cattle. Plasma samples were separated from the blood and stored frozen at -35° C.

Antisera

Three rabbits were immunized each with 60 µg of purified bovine factor H emulsified in Freund's complete adjuvant and injected subcutaneously. The rabbits were bled out 33 days after immunization. All rabbit sera had precipitating antibodies to bovine factor H as determined by double immunodiffusion and immunoelectrophoresis. Peroxidase-labelled goat anti-rabbit IgG was purchased from Organon Technica (Scarborough, Ont).

Analysis of bovine factor H polymorphism

Plasma proteins were separated by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) (Laemmli 1970) with the use of the Mini-PROTEAN II electrophoresis cell (Bio-Rad Laboratories [Canada] Ltd. Mississauga, Ont.). Plasma samples were diluted 1:10 in non-reducing buffer. Gel slabs, 1.5 mm thick, containing 6% polyacrylamide were loaded with 2 µl of plasma dilutions. After the electrophoretic run, electroblotting was carried out onto nitrocellulose sheets in the Trans-blot SD

semi-dry electrophoretic transfer cell (Bio-Rad) at 10 volts for 10 min. The nitrocellulose sheets were blocked with tris-buffered saline, pH 7.5, containing 3% gelatin. After washing, the sheets were incubated with rabbit anti-bovine factor H diluted 1:200 in tris-buffered saline containing 0.05% Tween 20 and 1% gelatin (TTBS-gelatin) for 45 min at room temperature (RT). After rinsing, the sheets were incubated with peroxydase-labelled goat anti-rabbit IgG, diluted 1:1000 in TTBS-gelatin, for another 45 min at RT. Finally, the nitrocellulose sheets were developed with HRP color development reagent (Bio-Rad).

Immunoprecipitation

After addition of EDTA (final concentration of 10 mM) to rabbit anti-bovine factor H, 300 µl of the antiserum was mixed with 100 µl of bovine plasma, incubated at RT for 30 min and subsequently spun at 13,000 x g. The precipitate was washed thoroughly and resuspended in 50 µl of SDS-buffer or SDS-buffer containing 5% 2-mercaptoethanol. Aliquots of 2 µl were run on SDS-PAGE (10% polyacrylamide) together with molecular weight markers in adjacent lanes.

Results

Electrophoretic polymorphism of bovine factor H

Polymorphism was demonstrated by the combined techniques of SDS-PAGE and immunoblotting. We have analyzed factor H in 149 plasma samples. All of them disclosed one of the three patterns of

factor H shown in Figure 1, i.e. three different phenotypes S, SF and F. The intensity of the bands in pattern SF appeared to be lower than the intensity of the corresponding bands in patterns S or F. Pattern SF could also be generated by mixing equal amounts of EDTA plasma of cattle showing pattern S and pattern F.

Family studies

Family studies demonstrated Mendelian segregation of S and F (Table 1). Our data indicate that these genetic variants are inherited by two codominant alleles at a single autosomal locus. We propose to name the locus RCA-H, with the two alleles RCA-H^F and RCA-H^S.

Population studies

Two populations of cattle were studied. The actual numbers of each phenotype in both the parental and offspring generation are given for each population in Table 2. The frequency of the allele S in the Holstein parents, Holstein offspring, crossbred parents and crossbred offspring were 0.50, 0.65, 0.75 and 0.62 respectively.

Molecular structure

Under non-reducing conditions the two genetic variants differed by about 6,000 daltons. Variant S was estimated to have a molecular weight of 135,000 daltons and variant F a molecular weight of 129,000 daltons (Figure 1). Under reducing conditions

immunoprecipitated variant S of bovine factor H had an apparent molecular weight of 169,000 daltons and variant F a molecular weight of 164,000 daltons (Figure 2). Thus, the two genetic variants differed in molecular weight by about 5,000 daltons under reducing conditions. When whole bovine serum or immunoprecipitated factor H were treated with 2-mercaptoethanol, the rabbit antiserum to bovine factor H did not detect factor H by immunoblotting. These results indicated that, after reduction of the disulfide bonds of factor H, the antigenic determinants, recognized by the rabbit antibodies, were destroyed.

All plasma samples showed an additional weaker staining band of an apparent molecular weight of 92,000 daltons under non-reducing conditions (Figure 1). Although a few samples had a double band at this position, there was no polymorphism that corresponded to the polymorphism of the major bands.

Discussion

Factor H has been isolated from mouse and man (Kinoshita & Nussenzweig 1984; Ripoche *et al.* 1988). The two predominant variants of human factor H have been ascribed to a T/C change in the structural gene for factor H, representing a tyrosine/histidine polymorphism at position 384 of the polypeptide (Day *et al.* 1988; Ripoche *et al.* 1988). There are no reports of differences between molecular weights of genetic variants of human factor H. We have found that the two described variants of bovine factor H differed in molecular weight by about 5,000 daltons. Human factor H is made up of 20 "short consensus repeats" (SCR) (Ripoche *et al.* 1988). The size of the SCR is 60 amino acids, which corresponds to about 6,000 daltons. This is about the size of the difference observed in the two variants of bovine factor H. Thus, the size difference of the two variants might represent an insertion or deletion of a SCR. At present, however, it is unknown whether the difference is due to a difference in the size of the polypeptide or a difference in the degree of glycosylation. In this connection it might be worthwhile to note that four allotypes of different molecular weights have been described for human CR1 which is structurally and functionally closely related to factor H (Klickstein *et al.* 1987).

Our molecular weight estimate of 135,000 daltons for unreduced allotype S matched well with that reported by Mhatre &

variants under reducing conditions (169 kd and 164 kd) were slightly higher than that (158 kd) obtained by Mhatre & Aston (1987). We believe that the discrepancies observed might be due to slight differences in technique. We identified the factor H isolated by Mhatre and Aston (1987) as allotype S by the SDS-PAGE/immunoblotting method (not shown).

The smaller protein of 92,000 daltons (Figure 1) detected by immunoblotting might be a protein entirely unrelated to factor H, a minor contaminant of the original factor H preparation. On the other hand, it might be a truncated form of factor H representing a translational product of a short factor H mRNA as has been described for human factor H (Fontaine *et al.* 1989).

The detection of polymorphism of bovine factor H might be used as an additional tool for parentage testing. In a blind study, we found that the phenotypes of the putative sire and those of several calves did not match. These mismatches were confirmed by other tests carried out in the Bovine Blood Typing Laboratory (not shown). More important, knowledge of genetic polymorphism of the proteins of the alternative complement pathway of cattle might be of potential value in predicting genetic resistance to infectious diseases.

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Acknowledgements

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Legends

Figure 1. Different electrophoretic patterns of bovine factor H demonstrated by SDS-PAGE and subsequent immunoblotting of EDTA plasma. Note: Besides the bands of allotypes S, SF and F, an additional band of lower molecular weight (92 kd) was demonstrated. It did not show a corresponding polymorphism.

Figure 2. Electrophoretic patterns of reduced bovine factor H of allotypes S and F. The three lanes contained molecular weight markers (MW), allotype S (S) and allotype F (F). The bands were obtained by immunoprecipitation of factor H from bovine plasma and electrophoresis of the immunoprecipitate on SDS-PAGE under reducing conditions. The gel was stained with Coomassie blue. Note: The apparent molecular weight was calculated to be 169 kd for allotype S and 164 kd for allotype F. The bands of 66 kd and smaller are believed to be the heavy chains of rabbit antibodies which made up part of the immune complexes applied to the electrophoresis gel.

Table 1. Inheritance of Factor H Types

Type		Number of Families	Offspring		
Sire	Dam		S	SF	F
S	S	1	1	-	-
S	SF	3	3	1	-
S	F	1	-	2	-
F	SF	2	-	2	-
F	F	1	-	-	1
SF	S	6	4	2	-
SF	SF	3	1	2	-
SF	F	1	-	-	1

TABLE 2. Phenotype Frequencies of Factor H

	Observed (%)			
Phenotype	F	FS	S	Total
Holstein parents	8(36.4)	6(27.3)	8(36.4)	22
Holstein offspring	3(17.6)	6(53.3)	8(47.1)	17
Crossbred parents	5(8.9)	18(32.1)	33(58.9)	56
Crossbred offspring	4(13)	27(50)	20(37)	54

F
F
S
SF
SF
S
SF
−
135 Kd
129 Kd
92 Kd
+

MW
S
F
200
116
92
66
45
169 Kd
164 Kd